Inserting membrane proteins in bacteria: characterizing the YidC and Sec substrate determinants

Senior Honors Thesis

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By

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Abstract

In this project, we investigated what determines whether a membrane protein inserts by the YidC/Sec-independent pathway, the YidC-only pathway, or the YidC/Sec pathway. Specifically, we wish to characterize the features of a membrane protein that determine path dependence. Our studies find that a membrane protein with a highly hydrophobic transmembrane segment inserts via the YidC/Sec-independent pathway. Addition of a negatively charged residue into the translocated periplasmic region or the transmembrane segment seems to switch insertion pathways to the YidC-only pathway, suggesting that charged residues in the translocated domain or transmembrane region is a YidC substrate determinant. On the other hand, the addition of a positively charged residue in the translocated domain or transmembrane of a membrane protein switches its insertion to the YidC/Sec pathway. We tested our studies initially using a single span membrane protein, and extended them to a protein that inserts with two hydrophobic domains. Our current findings indicate that that the YidC and YidC-Sec pathway have opposite charge requirements for membrane protein insertion.

Introduction

The assembly of proteins into biological membranes is an essential process for cell life. Once inserted, membrane proteins perform a multitude of physiological functions such as metabolite exchange and cell communication. Due to their vital roles in cell life, membrane proteins comprise over 50% of all drug targets (Dalbey et al., 2007).

To study membrane insertion processes, many labs employ the use of simpler prokaryotic systems to learn more about the complex membrane insertion events found in eukaryotic human cells. This can be done because the basic machinery for translocating proteins into or across membranes is remarkably conserved from *Escherichia coli* to humans (Samuelson et al., 2000).

In bacteria, most of the inner membrane proteins are membrane targeted cotranslationally by SRP and inserted by the SecYEG machinery. Proteins in bacteria that exhibited no dependence on SecYEG were thought to insert in the membrane without using protein translocase machinery. Then, in 2000, the Dalbey lab discovered YidC (Samuelson et al., 2000), a membrane protein insertion system that functions either with or without the Sec translocase (Dalbey et. al, 2007). Such a finding laid the foundation for three pathways for membrane protein insertion: a YidC/Sec pathway, a YidC only pathway, and a YidC/Sec-independent pathway (See Figure 1).

In *E. coli* some membrane proteins, such as KdpD, with a very hydrophobic transmembrane (TM) segment insert independently of the Sec and YidC machinery. It is thought, however, their insertion may yet be characterized by another mechanism involving an undiscovered protein (Facey and Kuhn, 2003). The majority of proteins in *E. coli*, nevertheless, are inserted by the YidC and Sec machinery, which work either independently or in concert to catalyze insertion.

In the YidC/Sec pathway, the Sec translocase is composed of the Sec translocation channel SecYEG and accessory proteins SecA, SecDFYajC, and YidC (see Figure 2) (Driessen and Nouwen, 2008). The SecA ATPase catalyses the translocation of hydrophilic domains by moving the domains through the SecYEG channel (Papanikou et al., 2007). Sec DFYajC aids in membrane protein insertion most likely by binding to the translocated domain of the inserting protein as it emerges on the periplasmic side of the membrane (Nouwen and Driessen, 2002; Tsukazaki et al.). In this way, SecDFYajC promotes the forward movement of the translocated region of the membrane protein. Finally, the third accessory protein is YidC, which plays a critical role for a subset of proteins. These include Subunit a of the F1Fo ATP synthase (Kol et al., 2009) and NuoK (Price and Driessen, 2009). YidC also aids the Sec machinery in the insertion and folding of the Sec dependent lac permease (Nagamori et al., 2004).

The second membrane insertion pathway is the YidC only pathway, where YidC inserts membrane proteins independently from the Sec translocase. Although in some instances, a portion of YidC is associated with the Sec translocase, much of it is free from the Sec machinery since it is much more abundant than the SecYEG channel (Urbanus et al., 2002). YidC, acting independently of the Sec translocase, inserts several crucial proteins, such as subunit c of the F1F0 ATP synthase (van der Laan et al., 2004), M13 Procoat (Sumuelson et al., 2000), and pf3 coat protein (Chen et al., 2002).

Finally, the third pathway is the YidC/Sec-independent pathway, which is when hydrophobic forces drive the insertion of the N-terminus of proteins such as leader peptidase into the membrane without the use of the YidC/Sec machinery (Samuelseon et al., 2000). As mentioned earlier, proteins that insert using the YidC machinery, such as Pf3 coat, can be made YidC independent by increasing the hydrophobicity of the transmembrane segment (Ernst et al., 2011).

A major question in the membrane field is what determines whether the YidC/Sec, YidC only or YidC/Sec independent pathway is used during membrane protein insertion. Does pathway selection depend on the overall hydrophobicity of transmembrane regions of protein substrates? Or is it related to the placement of charged residues in the transmembrane or translocated domain? One hypothesis comes from Driessen, who reported that YidC in conjunction with the Sec translocase was required for the insertion of subunit K of NADH:Ubiquinone Oxidoreductase (NuoK) when negatively charged residues were present in the transmembrane segments of NuoK (Price and Driessen, 2009). A second possibility is that YidC is needed for inserting membrane proteins with transmembrane segments that have an unfavorable distribution of positively charged residues around the TM segments (Gray et al. 2011). Finally, a third hypothesis is YidC is needed for insertion of proteins with a weak hydrophobic TM segment, which is based on the results that by increasing the hydrophobicity of the TM of YidC-dependent Pf3 coat, the protein inserts independently of translocation machinery (Ernst et al., 2011). Besides determining when YidC is required for insertion, it still also remains unclear when SecYEG is needed for insertion.

In this honor's thesis, we investigated the structural features of membrane proteins to begin identifying translocase determinants. We made mutations in a single span model membrane protein, followed by mutations in a protein that inserts with two hydrophobic domains, to understand what determines pathway selection for insertion. In determining pathway selection we could (1) identify YidC substrate determinants, (2) show that substrates with certain TM features and translocated regions must be handled by the Sec translocase, in addition to YidC, and (3) understand why the YidC/Oxa1/Alb3 family of proteins and the Sec translocase are essential to all organisms and have been conserved throughout evolution. We found that

addition of a charged residue into the periplasmic tail or polar residues in the TM segment can switch the insertion pathway from YidC/Sec-independent to the YidC or YidC/Sec dependent pathway. We also found the Sec translocase is required in conjunction with YidC when a positively charged residue is added to the TM or periplasmic domain.

Materials and Methods

Overview of Methodology

To change the structural features of membrane proteins, first site directed mutagenesis on the appropriate model membrane protein using the Quick change method was performed. The techniques in Maniatis et al. (Maniatis, 1982) were used for DNA manipulations in all experiments. DNA sequencing of the entire gene confirmed all mutations. The four model membrane proteins used in this study were the single span membrane proteins Pf3-LepTM, a-LepTM, and Pf3-aTM, and the model protein that inserts with two hydrophobic domains Procoat-Lep. The model proteins were cloned into the IPTG inducible pMS119 vector and expressed in the YidC depletion JS7131 strain, or cloned into the pLZ1 vector and expressed in the SecE depletion CM124 strain. After transforming plasmids into the appropriate strain, Proteinase K mapping was performed.

First IPTG induction is used to express the model membrane protein. Then cells were radio-labeled with [^{35}S]-methionine to detect the protein of interest. Cells were then converted into spheroplasts and Proteinase K was added to cleave all periplasmically exposed protein domains. A shift in the molecular weight of the model membrane protein upon adding Proteinase K indicates the inner membrane protein inserted across the membrane. Then Immunoprecipitation was performed to isolate the model membrane protein. Finally, the results were analyzed using SDS Page and phophorimaging. (See Figure 3 for an overview of methodology)

Strains and growth conditions

The YidC depletion JS7131 strain was from our lab's collection. The SecE depletion CM124 strain was from Beth Traxler and is described in (Traxler and Murphy, 1996). *yidC and secE* genes in the JS7131 and CM124 strains are under the araBAD promoter.

JS7131 cells were cultured at 37°C for 3 h in LB media with 0.2% Arabinose (YidC expression conditions) or 0.2% glucose (YidC depletion conditions) (Samuelson et al., 2000). The SecE depletion strain CM124 was cultured in M9 media supplemented with 0.2% Arabinose with 0.4% glucose (SecE expression conditions) or 0.4% glucose (SecE depletion conditions) for 9 h at 37°C (Traxler and Murphy, 1996). Prior to induction of the plasmid-encoded proteins in JS7131 and CM124, the media was exchanged to M9 media (Miller, 1972) containing 0.5% fructose and 50 µg/mL of each amino acid except Methionine, and shaken for 30 min at 37°C.

Protease-accessibility Assay

Expression of the constructs was induced by 1 mM IPTG for 3 min, and cells were labeled with [^{35}S]-methionine for 1 min. Cells were then converted to spheroplasts (Delgado-Partin and Dalbey, 1998). The [^{35}S]-labeled cells were then treated with Proteinase K (0.75 mg/mL) for 1 h. After inactivating the Proteinase K with 5 mM PMSF, the cells were precipitated with 20% Tricholoracetic acid. The cell pellet was washed with ice cold acetone and solubilized with SDS-Tris buffer (10 mM Tris-HCl, pH 8.0, 2% SDS). (See Figure 4 for a model of the Protease-accessibility Assay)

Immunoprecipation

Samples in SDS-Tris buffer were diluted in Triton X-100 Tris buffer and immunoprecipitated with antiserum to leader peptidase, which precipitates the Lep derivatives. All model membrane proteins have a leader peptidase C-terminal domain that acts as a tag. The samples were also immunoprecipitated with antiserum to OmpA (Outer Membrane Protein A), which serves as a positive control for the spheroplasts formation. OmpA is not digested in intact *E. coli* but is digested in spheroplasts.

Analysis of YidC or SecE dependence results

The samples were analyzed by SDS-PAGE and phosphorimaging. The radioactive dried SDS PAGE gels were scanned using a Typhoon Trio Scanner. (See Figure 5 for SDS PAGE recipes).

Materials

Phenylmethlysulfonyl fluoride and lysozyme were from Sigma. Proteinase K was purchased from Qiagen. IPTG was from Research Products International Corporation. Trans [^{35}S]-label, a mixture of 85% [^{35}S]-methionine and 15% [^{35}S]-cysteine, 1000 Ci/mmol was from ICN. Antiserum to leader peptidase (anti-Lep) and OmpA (anti-OmpA) were from our own laboratory collection.

Results

The N-Tail and the TM segment determine the insertion pathway

To examine what features of a membrane protein determine the pathway used, we initially studied three model membrane proteins that insert with a single hydrophobic domain. We then later extended our studies to a fourth model membrane protein that inserts with two hydrophobic domains. The model proteins differ only in the translocated N-tail region and/or TM segment. The constructs are named with the first part representing the N-tail and the second part representing the TM. Pf3-LepTM contains the Pf3coat periplasmic tail region attached to the LepTM. a-LepTM contains the Foa (subunit a of the F1Fo ATP synthase) periplasmic tail region fused to the N-terminus of Lep like the Pf3-LepTM construct. The third model protein Pf3-aTM contains the Pf3 coat periplasmic tail region fused with the TM1 of Foa (See Figure 6 for the three single hydrophobic domain model membrane proteins and Figure 7 for their amino acid sequences). All of the membrane proteins that insert with a single hydrophobic domain contain an Arginine introduced after TM2 to prevent the C-terminus from being translocated across the membrane (Cao and Dalbey, 1994). The addition of the Arginine residue after TM2 of Lep simplifies the Proteinase K assays by allowing only the translocation of the N-terminal tail to be assayed since it is the only region translocated across the inner membrane.

The YidC dependence of the model membrane proteins was assayed using the methods described above in Methodology. While Subunit a of F1Fo ATP synthase (Kol et al., 2009) and pf3 coat protein (Samuelson et al., 2001) were known to be YidC dependent, Lep was found to be YidC independent (Samuelson et al., 2000). When the Pf3 Coat N-tail was attached to Lep TM1, the protein was found to be YidC independent. The challenge then became to investigate YidC

determinants. When the Pf3 coat N-tail was switched for the N-tail of Foa, a-LepTM was found to be YidC dependent, indicating the N-tail of Foa was a YidC determinant. When the LepTM of Pf3-LepTM was switched for TM1 of Foa, Pf3-aTM was found to be YidC dependent, indicating the TM is also a YidC substrate determinant (See Figure 8 for a sample full length SDS PAGE gel with an explanation of how to determine YidC dependence from the gel, and Figure 9 for the results of the three model membrane proteins indicating N-tail and TM domains as determinants for dictating insertion pathways). Here degradation of outer membrane protein A (OmpA) was used as a positive control to indicate efficiency of spheroplasts formation, as it is not digested in intact cells. OmpA is completely digested in all Proteinase K + studies showing conversion to spheroplasts (Figure 9, right panels).

Addition of a charged residue in the N-tail determines YidC dependency

Comparing the N-tail of Foa to the N-tail of Pf3-LepTM (See Figures 6 and 7) indicates that there are more negatively charged residues in the N-tail of Foa. To test whether negatively charged residues determine YidC dependency, we tested Pf3-Lep V15D, which is when the Val at residue 15 in the N-tail was mutated to negatively charged Asp. The JS7131 cells expressing the Pf3-Lep V15D were grown under YidC expression and depletion conditions. As seen in Figure 10, the PK digestion of the N-tail did not occur under YidC depletion conditions, but did occur under YidC expression conditions. Additional studies also found that with a doubly negatively charged mutant for Pf3-Lep TM V4E/V15D was also YidC dependent (Zhu et al., 2013). We also tested if there was any difference in terms of YidC dependence if the charge was negative or positive. Upon testing the mutant Pf3-LepTM V15R, it was found that YidC was still needed for insertion (See Figure 10). Subsequent studies have shown that additional positive charges block insertion due to acting as a barrier for translocation according to the

positive inside rule (Zhu et al., 2013). These results show that adding either a negatively or positively charged residue to the N-tail can make the protein YidC dependent for insertion.

Decreased hydrophobicity in the TM segment indicates YidC dependency

We also tested charged residues located in the TM segment as a YidC substrate determinant since YidC dependent Pf3-aTM has an unfavorable negatively charged residue within its TM segment, while Pf3-LepTM does not. By adding a positively charged Arg in position 23 in place of the Ala in the Pf3-LepTM transmembrane, the mutant was changed from YidC independent to YidC dependent, as indicated by the shift in bands with Proteinase K digestion under YidC expression conditions, but not under YidC depletion conditions in Figure 11. On the other hand, by removing a negatively charged residue in the TM of Pf3-aTM D23A, where Ala replaced the negatively charged Asp, the mutant was made YidC independent. Strikingly, upon substitution of the charged residue in place of a polar residue as in Pf3-aTM D23N, the substrate remained YidC dependent. Finally, the position of the charged residue in the TM did not seem to affect YidC dependency, as can be seen from Pf3-aTM, D23A/F27R, where the positively charged residue was moved further down the TM segment (Figure 11). The results show that not just negative or positive charges in the TM dictate YidC dependency, but so do polar residues. Additionally, where they are placed in the TM does not seem to change YidC dependency. Subsequent positional scanning studies with Pf3-aTM, where charged or polar residues were placed throughout the membrane, have confirmed this result (Zhu et. al, 2013). These results show decreased hydrophobicity is a YidC substrate determinant.

The Sec translocase is required to translocate the N-tail or TM of a protein when a positively charged residue is introduced, switching insertion to the YidC/Sec pathway.

All of the membrane protein mutants that were tested for YidC dependence, were subsequently also tested for Sec dependence (Figure 12), using the CM124 SecE depletion strain as described in the methodology. It has been well established that proteins requiring SecYEG machinery are inhibited from being translocated across the membrane when SecE has been depleted (Traxler and Murphy, 1996). SecE depletion leads to degradation of SecY. Aside from growth conditions, the Proteinase K mapping assay is the same as with the JS7131 YidC depletion strain.

When a negative charge was introduced in the N-tail, as in Pf3-LepTM V15D, or when a positive charge was removed from the N-Tail as in a-LepTM R24A, the mutant membrane proteins were still found to be Sec independent (Figure 12). On the other hand, if a positive charge was introduced in the N-tail, as in Pf3-LepTM V15R, it was found the membrane protein would require the Sec translocase.

When a positive charge was introduced into the transmembrane region, as in Pf3-LepTM A23R, it was found that the Sec translocase was also required in this case. The position of the positive charge also did not seem to change the Sec dependence, as Pf3-aTM D23A/F27R still remained Sec dependent. However, upon introducing a neutral slightly hydrophobic or even polar charged residue in place of the negatively charged residue in the transmembrane in Pf3-LepTM D23A and Pf3-LepTM D23N, the membrane protein still remained Sec independent (Figure 12). Such results seem to indicate that the Sec translocase, in addition to YidC, is required to translocate an N-tail or transmembrane region with a positive charge.

Extension of results to a model membrane protein that inserts with two hydrophobic domains seems to confirm previous data

We next extended our studies to test whether a positive charge added in different places with a protein that inserts with two hydrophobic domains, ProCoat-Lep, still leads to the Sec-dependent membrane insertion pattern observed before. Figure 13 shows a model of how M13 ProCoat-Lep inserts into the membrane and Figure 14 shows a sample SDS-Page gel and how to interpret results. As shown in Figure 16, ProCoat-Lep WT is a YidC dependent, Sec-independent protein.

To test whether Sec dependence relied on a positive charge in the periplasmic domain, as it did in the previous N-tail studies, we made mutations (highlighted in red in the amino acid sequence of ProCoat-Lep in Figure 15) in the periplasmic loop region of the amino acid sequence AEGDD. It was found that upon mutating AEGDD to ARGNN, which contains an additional positive charge in the periplasmic domain, only a very slight Sec dependence was seen (See Figure 17). However, in the mutants ANGRR (two positive charges) and ARGRR (three positive charges), even greater Sec dependence was seen to the point where ARGRR was nearly completely Sec dependent (Figure 17). As expected, all of the mutants were YidC dependent, since the parent ProCoat-Lep is strictly dependent upon YidC for insertion.

Further studies will need to be completed to test whether the addition of a positive charge in the transmembrane region of ProCoat-Lep dictates Sec dependence.

Discussion

This paper studied the insertion of four model membrane proteins, each of which had a periplasmic N-tail and TM segment. We examined the structural features of the proteins to determine whether they insert by the YidC/Sec-independent, YidC-only, or YidC/Sec-dependent pathways. Mutations made within the model protein N-tail and TM regions helped us understand what structural features determine pathway selection for insertion. Three key observations from our studies can be made. One, a single charged residue in the N-tail region can switch the insertion pathway for membrane proteins from YidC independent to YidC dependent in *E. coli*. Two, a weakly hydrophobic TM segment is also a YidC substrate determinant. And three, there are opposite charge requirements for the YidC and YidC/Sec dependent pathways. A membrane protein with a negative charge in the N-tail or TM segment seems to insert via the YidC pathway, whereas a protein with a positively charged residue inserts by the YidC/Sec pathway.

Supporting the first observation, that negatively charges residues in periplasmic domains are a YidC substrate determinant, is that many currently known YidC substrates contain negatively charged residues in their periplasmic regions. For example, the YidC dependent substrates M13 ProCoat, Pf3 coat, subunit a and c (of F1Fo ATP synthase) all have negatively charged residues in their periplasmic regions with few to no positively charged residues (Fillingame et al., 2000; Price and Driessen, 2009). The YidC homolog in mitochondria, Oxa1, which aids in inserting proteins into the mitochondrial inner membrane from the matrix, also aids in translocating a large number of proteins with many negative charges (van der Laan et al., 2004). Our results, however, also point out that the bacterial YidC is needed to insert proteins with a positive charged residue in the translocated region.

The second observation, that a weakly hydrophobic TM segment is a YidC substrate determinant, is also consistent with the known YidC-dependent substrate NuoK that has a negatively charged residue in its TM segment (Price and Driessen, 2009). Our studies also show that a weakly hydrophobic TM region is an important feature for determining YidC as a requirement for insertion. Either a positive or negative charge introduced into a transmembrane segment makes the protein strictly YidC dependent. This is different from the recent study suggesting that YidC dependency for NuoK insertion was based only on the presence of two negatively charged residues in the TM segment, and not on the presence of positively charged residues (Price and Driessen, 2009).

Finally, our studies have shown the third observation, that there are opposite charge requirements for the YidC and YidC/Sec dependent pathways. YidC seems to have limited translocation capacity, since it seems to insert N-tail and TM regions with positively charge residues only in concert with the Sec Translocase. A scenario that has been put forth is YidC binds to the inserting membrane protein during membrane partitioning, and then the SecYEG channel binds to the TM segment of the membrane protein to promote its translocation (Zhu et al., 2013). The Sec translocase requirement seems to coincide with the Sec-dependent insertion we saw for the a-LepTM, Pf3-LepTM, and ProCoat-Lep constructs which contain the added positively charged residues in this project. We found that the Sec translocase was not needed for membrane insertion for several negatively charged TM or N-tail mutants, but was needed when a positively charged residue was introduced.

As for a final discussion point, a membrane protein with a highly hydrophobic TM segment has the capacity to insert without the YidC or Sec translocase because the hydrophobic force of the TM is sufficient to drive membrane translocation. With added negative or positive charges to the

N-tail, insertion requires YidC or YidC/Sec, as the hydrophobic force becomes insufficient to translocate the polar N-tail region. Similarly, adding a polar or charged residue in the TM prevents insertion spontaneously because the hydrophobic force of this region is reduced, therefore requiring YidC or YidC/Sec for insertion.

Further studies will continue to shed light on the membrane protein insertion pathways that exist in bacteria. Certainly additional mutants from the constructs in the project would help further clarify this project's results. Specifically, further TM mutants using the ProCoat-Lep model protein would aid in understanding Sec translocation. Eventually, maybe even proteins that seem to insert spontaneously into the membrane may be discovered to use an alternate membrane protein insertion path. Only with continuous investigation will we know such findings.

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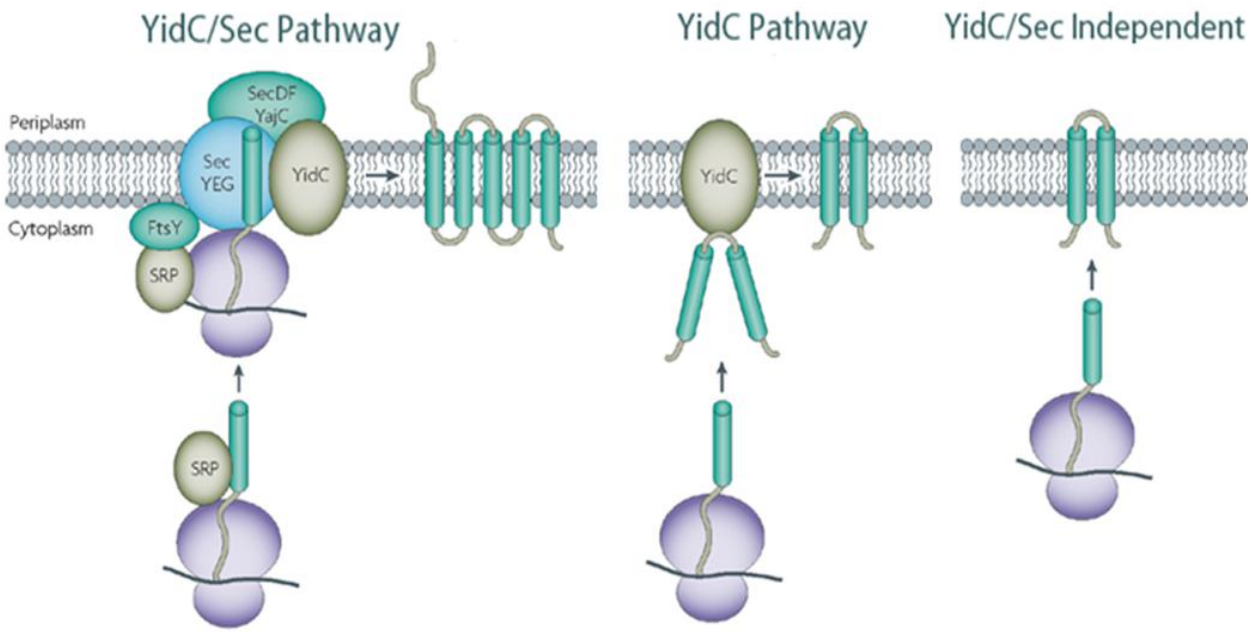

Figure 1: Membrane Insertion Pathways. Left: YidC acting in concert with the Sec translocase to insert an inner membrane protein. Middle: YidC independently inserting a membrane protein. Right: hydrophobic force of the TM segment is sufficient enough to drive insertion spontaneously.

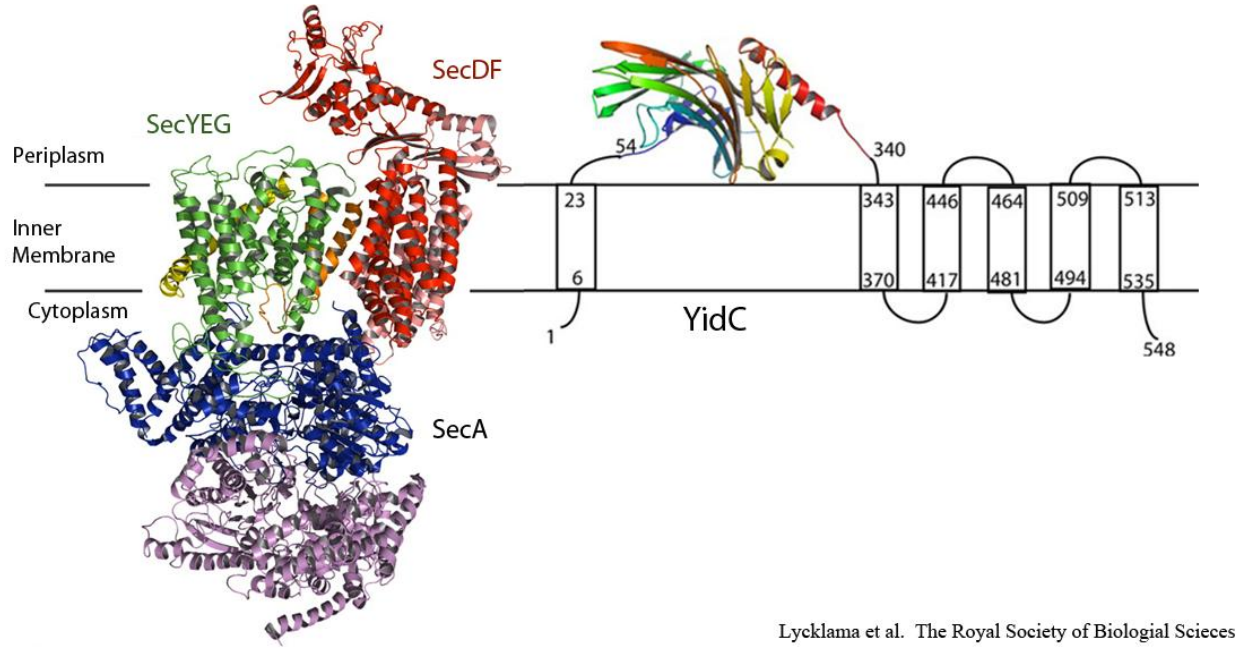

Figure 2: Structure of the YidC Sec Translocase with its accessory proteins.

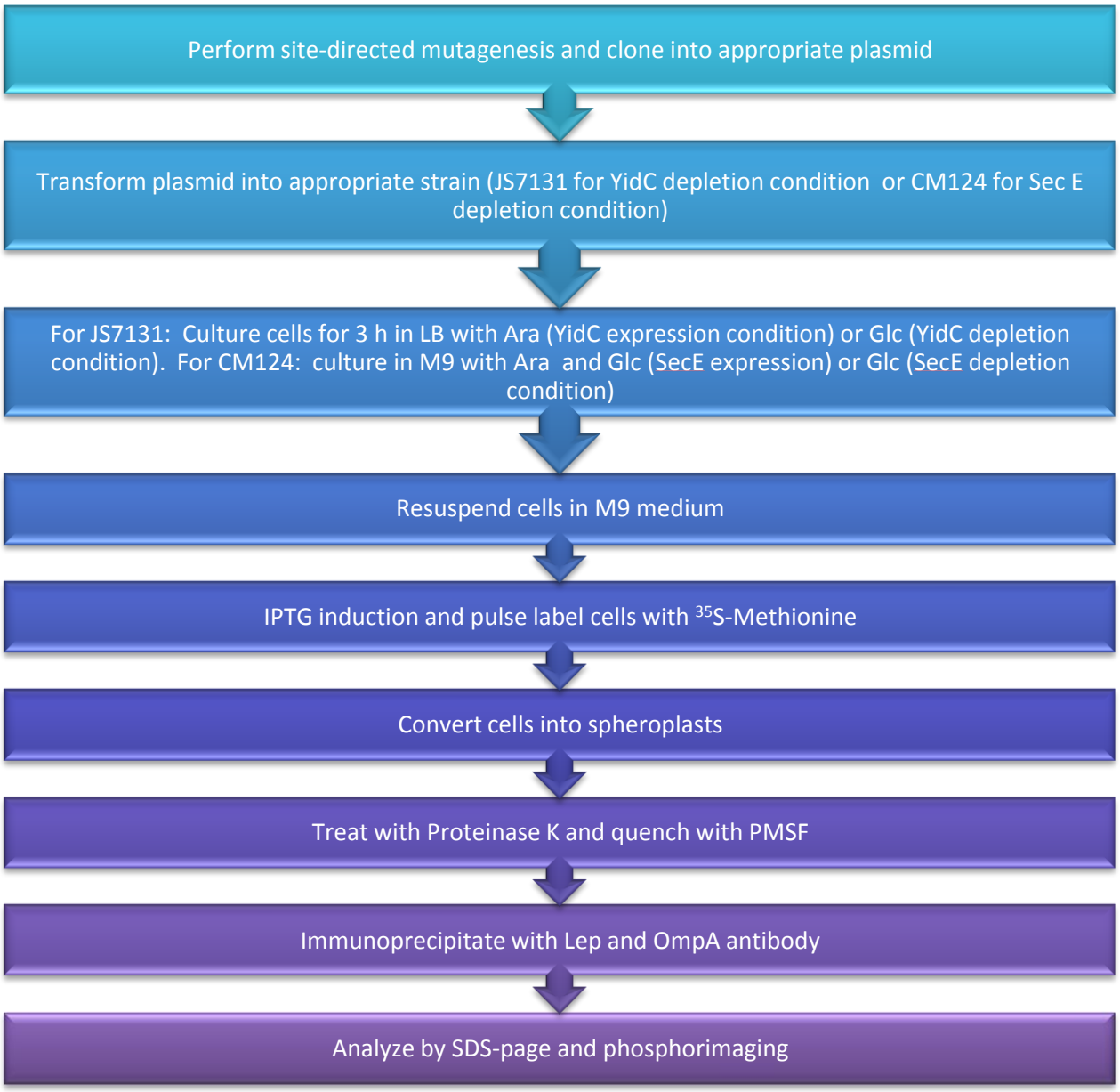

Figure 3: Overview of Methodology. A simplified step by step protocol of the methods used in determining YidC and Sec dependency for model membrane proteins.

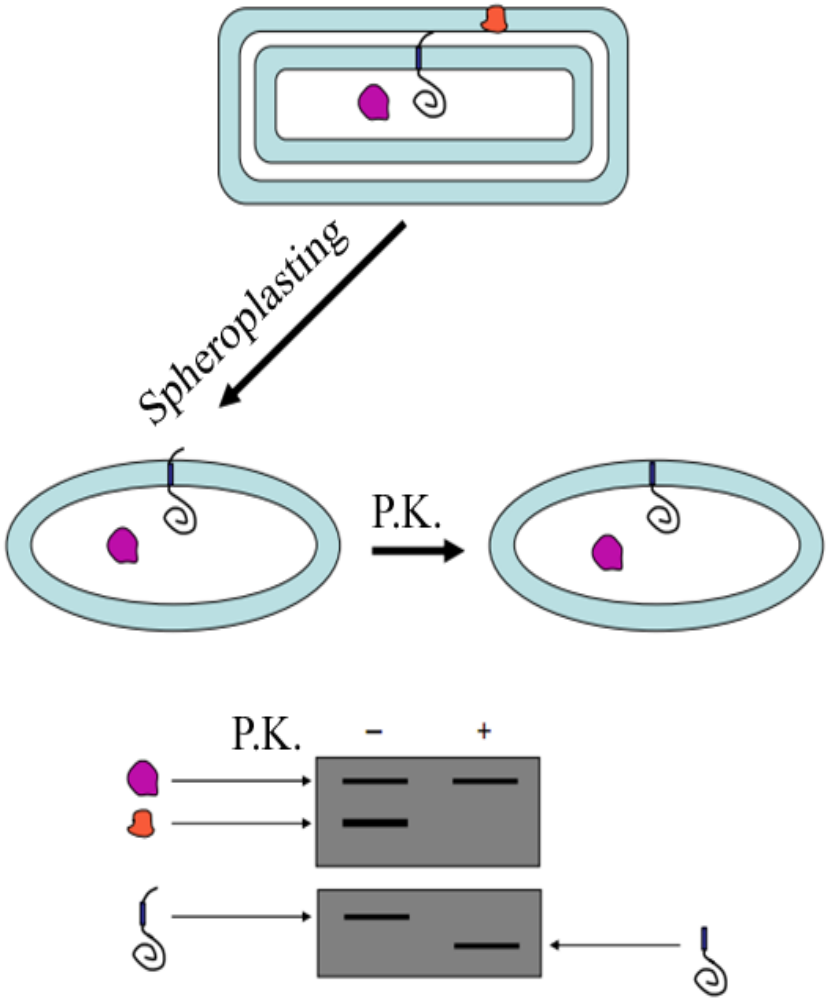

Figure 4: Proteinase K Accessibility Assay: Periplasmically exposed protein domains are cleaved by proteinase K while cytoplasmic proteins and nontranslocated domains of membrane proteins remain protected within spheroplasts. The difference in molecular weight after cleavage by Proteinase K can be detected on an SDS PAGE gel.

Recipe for 15% SDS Page Gels

Reagents	15% SDS Running Gel:	15% SDS Stacking Gel
dH_2O	1208 µL	819 µL
0.5 M Tris-HCl SDS, pH 6.8	0 µL	131 µL
1.5 M Tris-HCl SDS, pH 8.8	945 µL	0 µL
40% Acrylamide	1313 µL	337.5 µL
10% Ammonium Persulfate	35 µL	12.5 µL
10 λ TEMED (N, N, N', N' – Tetramethyl ethylene diamine)	1.5 µL	1.25 µL

Figure 5: 15% SDS Gel Recipe. SDS Page gels were used to determine whether a shift in the molecular weight of the model membrane protein occurred upon adding Proteinase K.

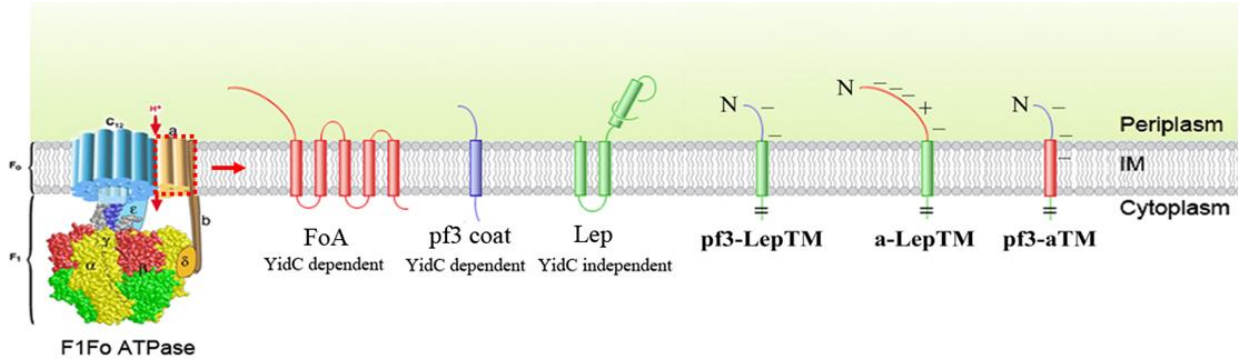

Figure 6: The topology of the three model membrane proteins Pf3-LepTM, a-LepTM, Pf3-aTM that insert with a single hydrophobic domain. The four membrane proteins on the left (F1Fo ATPase, FoA, Pf3 Coat, and Lep) indicate where the model membrane proteins were derived from. The model membrane protein Pf3-LepTM has the N-tail of Pf3 Coat, and the TM1 of Lep. a-LepTM has the N-tail of FoA and the TM1 of Lep. Pf3-aTM has the N-tail of Pf3 Coat and the TM1 of FoA.

The N-tail and TM amino acid sequences of the three model proteins:

pf3-LepTM: MQSVITDVTGQLTAVQAD**ANMFALILVIATLVTGILWCV**

a-LepTM: MASENMTPQDYIGHHLNNLQLDLRTFSLVDPQNPPATFW**ANMFA LILVIATLVTGILWCV**

pf3-aTM: MQSVITDVTGQLTAVQAD**TINIDSMFFSVVLGLLFLVLF**

Note: Sequences bolded and underlined are in the TM. Residues in red were mutated in the study.

Figure 7: Amino acid sequences of the three model membrane proteins that insert with a single hydrophobic domain.

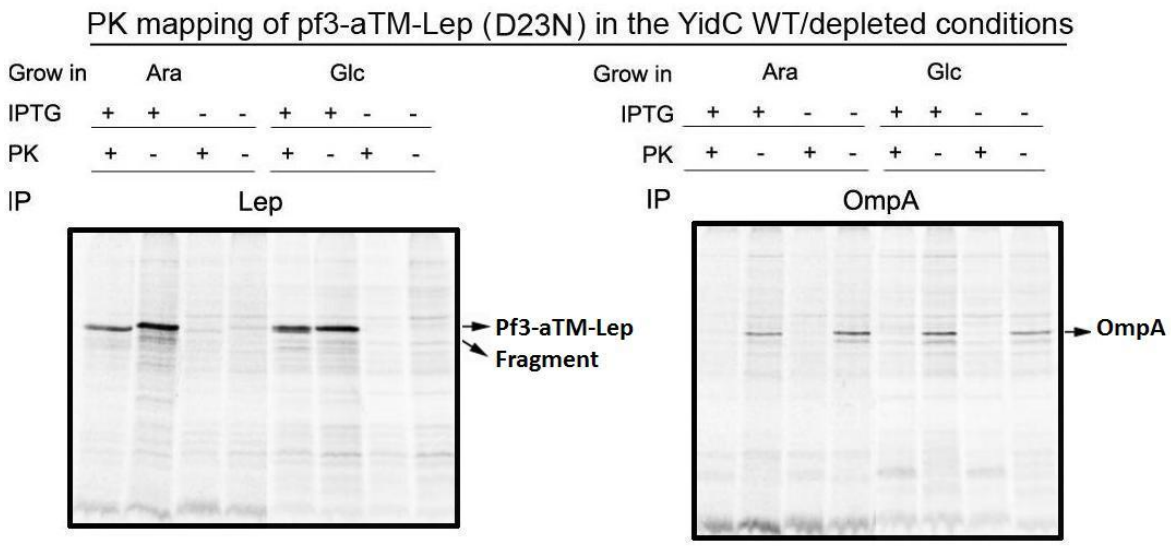

Figure 8: Sample full length SDS Page Gel. The model membrane protein here is Pf3-aTM with a Lep tag and mutation D23N. A shift in the left two columns under Lep Ara (YidC expression) but a lack of a shift under Lep Glc (YidC depletion) indicates that the protein is YidC dependent. Fragment indicates Proteinase K had access to the inserted membrane protein. IPTG + induces expression of the protein of interest Pf3-aTM (D23N), and IPTG – is a negative control; no major bands should be in those latter columns. OmpA indicates spheroplast formation and if YidC depletion had a negative impact by depleting the proton-motive-force too much. The precursor form of OmpA called Pro-OmpA would accumulate on the gel if the pmf was over depleted, since OmpA is pmf-dependent for export across the inner membrane.

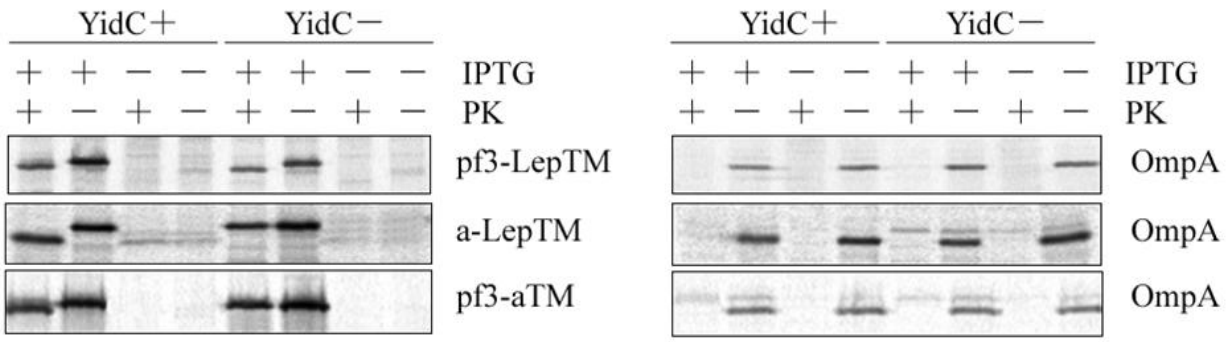

Figure 9: The N tail and TM segment can function as a YidC substrate determinant. *E. coli* JS7131 cells bearing the plasmids of the three different model membrane proteins were grown under YidC expression or YidC depletion conditions, labeled, and analyzed for translocation of the N-tail using Proteinase K accessibility assay described under methodology. The plasmids encoded Pf3-LepTM, a-LepTM, and Pf3-aTM. Pf3-LepTM was found to be YidC independent, as indicated by the shift under both YidC expression and depletion conditions. a-LepTM and Pf3-LepTM, on the other hand, were both YidC dependent.

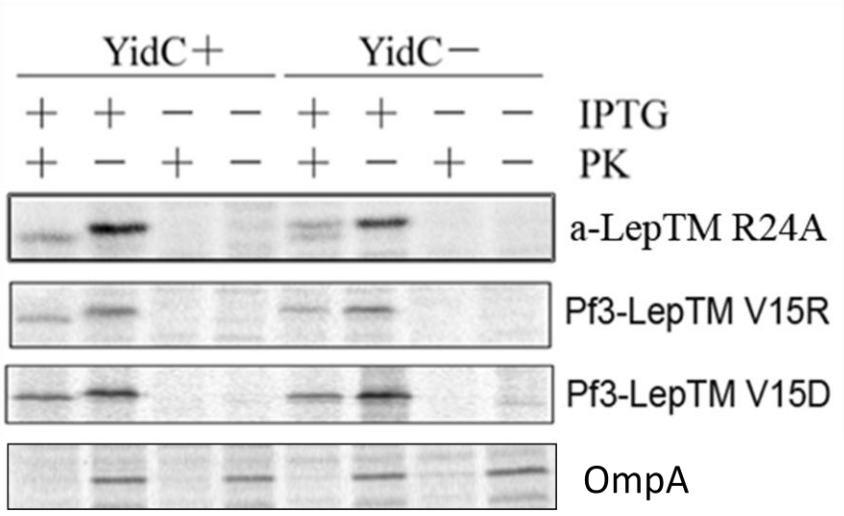

Figure 10: Addition of a charged residue in the N-tail determines YidC dependency. JS7131 expressing different plasmids was analyzed for N-tail translocation using the Proteinase K mapping assay described under methodology. The plasmids encoded the proteins a-LepTM (R24A), Pf3-LepTM (V15R), and Pf3-LepTM (V15D). Both Pf3-Lep V15R and Pf3-Lep V15D were YidC dependent. Upon mutating Arg for Ala in the N-tail for a-LepTM (resulting in a-LepTM R24A), there was less YidC dependence for the mutant, which can be seen by the shift in the YidC – conditions. OmpA is a positive control that looks the same for all three mutants.

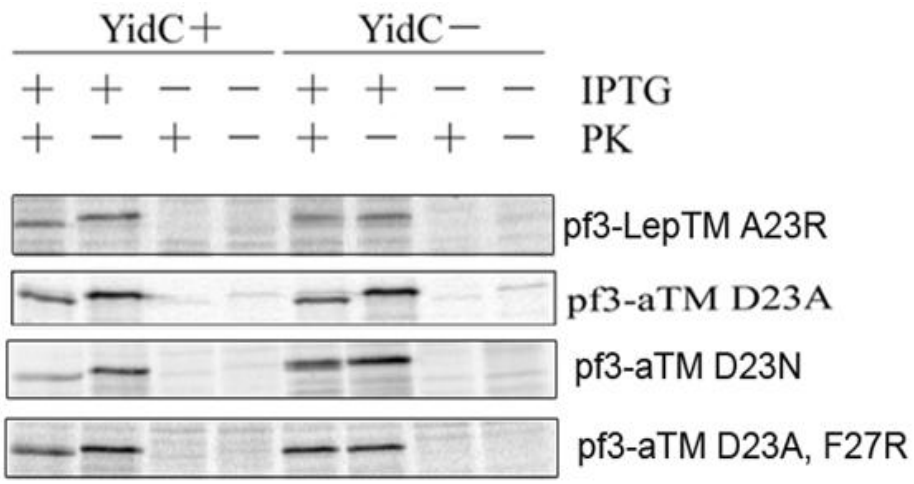

Figure 11: Decreased hydrophobicity in the TM segment results in YidC dependency for insertion. *E. coli* JS7131 cells expressing different plasmids were analyzed by Proteinase K mapping assay described under methodology. The plasmids expressed the proteins Pf3-LepTM (A23R), Pf3-aTM (D23N), Pf3-aTM (D23A, F27R) and Pf3-aTM (D23A). For Pf3-LepTM (A23R), Pf3-aTM (D23N), and Pf3-aTM (D23A, F27R), YidC dependence can be observed. However, Pf3-aTM (D23A) is YidC independent, as seen by the shift observed under both YidC expression and depletion conditions.

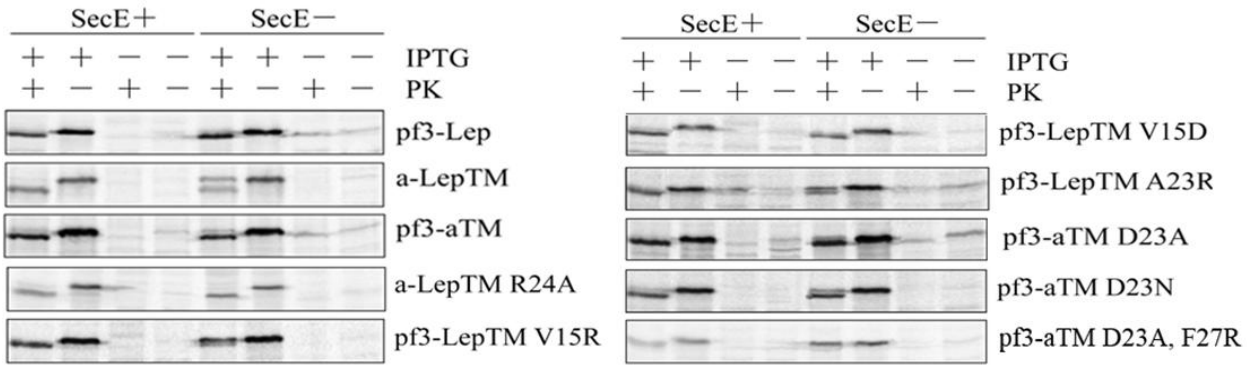

Figure 12: The Sec translocase is required to translocate the N-tail of a protein when a positively charged residue is introduced in the tail or TM segment, switching insertion to the YidC/Sec pathway. *E. coli* CM124 cells expressing different plasmids were grown under SecE expression and depletion conditions and analyzed by the Proteinase K mapping assay described under methodology. The first three proteins in the left hand column, Pf3-LepTM, a-LepTM, and Pf3-aTM, are the parent constructs. The remaining proteins are mutant model constructs. The plasmids expressed a-LepTM (R24A), Pf3-LepTM (V15R), Pf3-LepTM (V15D), Pf3-LepTM (A23R), Pf3-aTM (D23A), Pf3-aTM (D23N), and Pf3-aTM (D23A, F27R). Note: The Sec data figures for Pf3-LepTM, a-LepTM, Pf3-aTM, and their mutants are generously provided by the project mentor Dr. Lu Zhu.

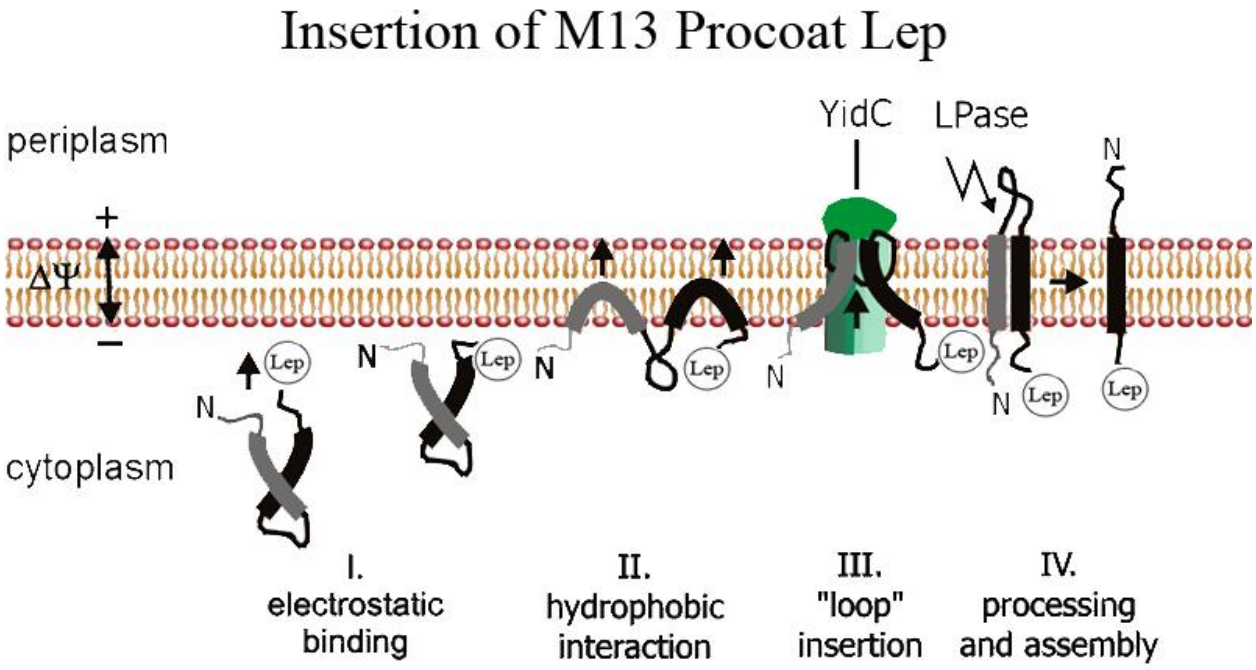

Figure 13: Model of how ProCoat-Lep inserts into the membrane. Upon YidC-catalyzed insertion, ProCoat-Lep gets cleaved by Leader Peptidase. This generates the mature coat protein called Coat-Lep, which is seen on the very right hand side of the figure. Coat-Lep is then what Proteinase K digests if ProCoat-Lep was inserted into the membrane.

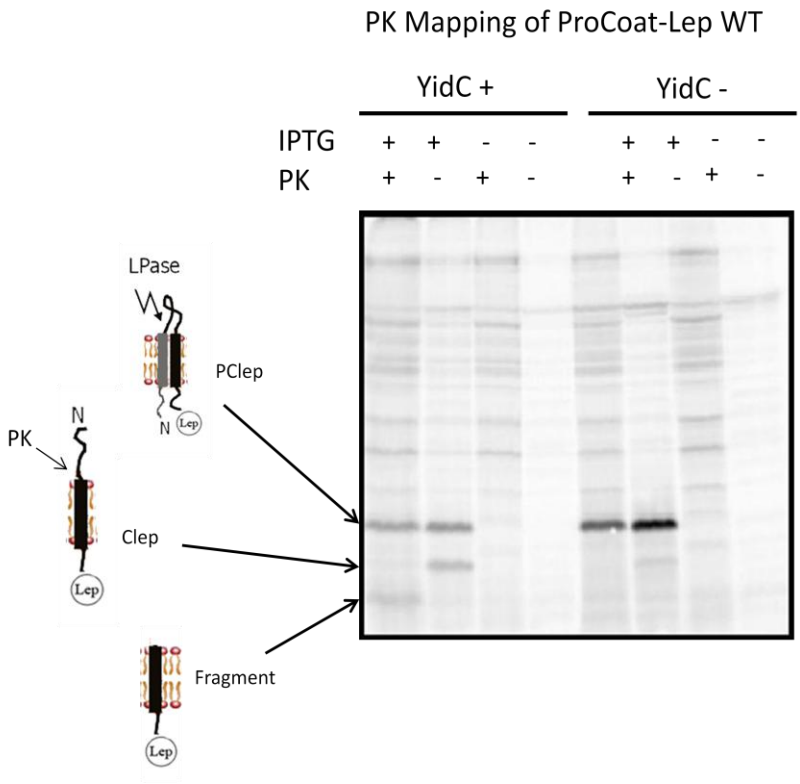

Figure 14: A sample SDS PAGE Gel for ProCoat-Lep. The model membrane protein here is ProCoat-Lep, wildtype. A shift in the left two columns under Lep Ara (YidC expression) but a lack of a shift under Lep Glc (YidC depletion) indicates that the protein is YidC dependent. Fragment indicates Proteinase K had access to the inserted PClep protein. (See Figure 13 for an explanation of how ProCoat-Lep inserts). IPTG + induces expression of the protein of interest ProCoat-Lep and IPTG – is a negative control; no major bands should be in those columns.

The two hydrophobic segments and periplasmic loop amino acid sequence of ProCoat-Lep:

MKKSLVLKASVAVATLVPMLSFAAEGDDPAKAAFNSLQASATE**YIGYAWAMVVVI VGATIGIKLFKKFTSK**

Note: Sequences bolded and underlined are in the hydrophobic segments. Residues in red were mutated in the study.

Figure 15: Amino acid sequence of ProCoat-Lep.

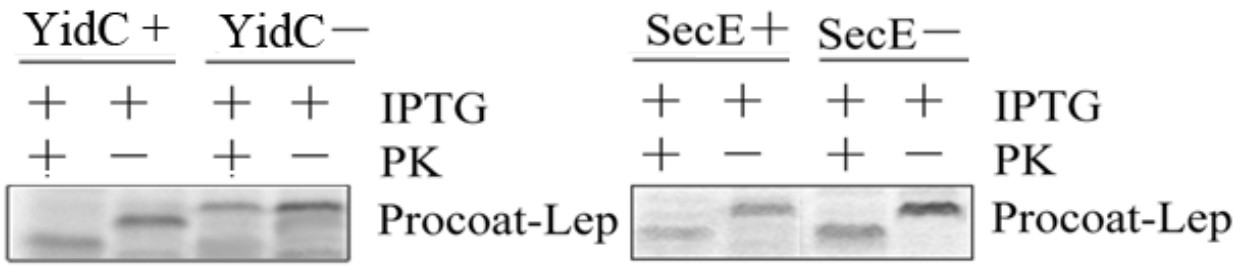

Figure 16: YidC and Sec dependence of ProCoat-Lep WT. Left: *E. coli* JS7131 cells expressing ProCoat-Lep WT were analyzed by Proteinase K mapping assay described under methodology. ProCoat-Lep was found to be YidC dependent. Right: *E. coli* CM124 cells expressing ProCoat-Lep WT were grown under SecE expression and depletion conditions and analyzed by the Proteinase K mapping assay described under methodology. ProCoat-Lep was found to be Sec independent. Note: Only IPTG+ conditions are shown here.

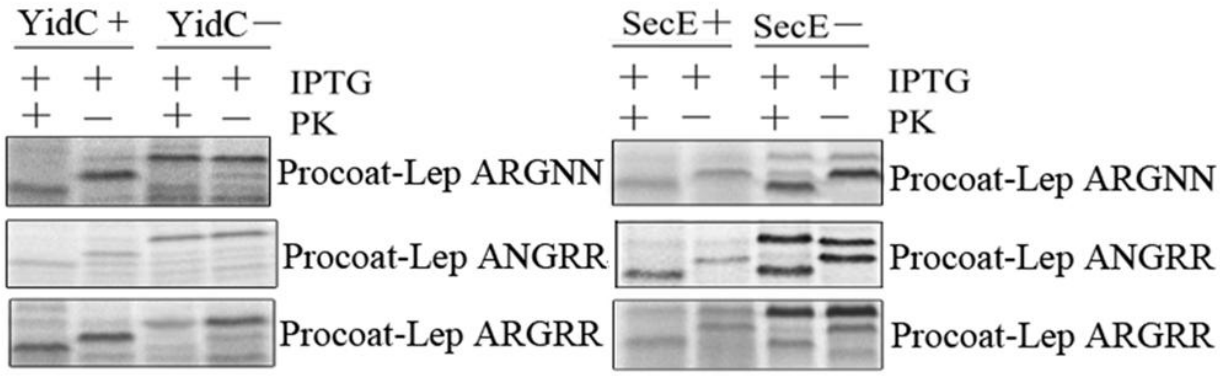

Figure 17: Positive charges in the periplasmic loop of ProCoat-Lep seem to be consistent with the YidC/Sec dependent pathway. Left: *E. coli* JS7131 cells expressing different plasmids were analyzed by Proteinase K mapping assay described under methodology. The plasmids expressed the proteins ProCoat-Lep (ARGNN), ProCoat-Lep (ANGRR), and ProCoat-Lep (ARGRR). Right: *E. coli* CM124 cells expressing different plasmids were grown under SecE expression and depletion conditions and analyzed by the Proteinase K mapping assay described under methodology. The plasmids expressed the proteins ProCoat-Lep (ARGNN), ProCoat-Lep (ANGRR), and ProCoat-Lep (ARGRR).